

AD-A171 387

DEVELOPMENT OF SEROLOGIC ASSAYS FOR THE DIAGNOSIS OF  
NEW WORLD LEISHMANIASIS(U) MARYLAND UNIV BALTIMORE DEPT  
OF PATHOLOGY R L ANTHONY 31 JAN 84 DAMD17-83-C-3831

1/1

UNCLASSIFIED

F/G 6/3

NL

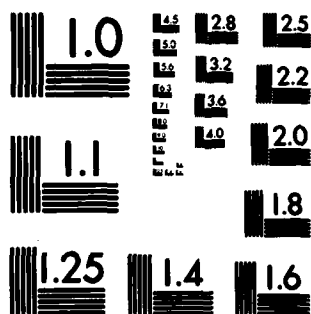
END

DATE

FILED

10-86

(DT)



MICROCOPY RESOLUTION TEST CHART  
NATIONAL BUREAU OF STANDARDS-1963-A

AD-A171 387

DTIC ACCESSION NUMBER

LEVEL

PHOTOGRAPH THIS SHEET

REPORT No. 1B

DEVELOPMENT OF SEROLOGIC  
ASSAYS FOR THE DIAGNOSIS OF

NEW WORLD LEISHMANIASIS

ANNUAL REPORT (12-1-82 / 11-30-83)

INVENTORY

Jan. 31, 1984

DOCUMENT IDENTIFICATION

**DISTRIBUTION STATEMENT A**

Approved for public release  
Distribution Unlimited

DISTRIBUTION STATEMENT

ACCESSION FOR

NTIS GRA&I ☒

DTIC TAB ☐

UNANNOUNCED ☐

JUSTIFICATION

BY

DISTRIBUTION /

AVAILABILITY CODES

DIST

AVAIL AND/OR SPECIAL

A-1

DISTRIBUTION STAMP

QUALITY  
INSPECTED  
4

DTIC  
ELECTE

SEP 03 1986

D

DATE ACCESSIONED

DATE RETURNED

REGISTERED OR CERTIFIED NO.

DATE RECEIVED IN DTIC

PHOTOGRAPH THIS SHEET AND RETURN TO DTIC-DDAC

AD-A171 387

REPORT NUMBER 1B

DEVELOPMENT OF SEROLOGIC ASSAYS FOR THE DIAGNOSIS  
OF NEW WORLD LEISHMANIASIS

ANNUAL REPORT (DECEMBER 1, 1982-NOVEMBER 30, 1983)

Ronald L. Anthony, Ph.D.  
Department of Pathology

January 31, 1984

US ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND  
Fort Detrick, Frederick, Maryland 21701

Contract No. DAMD 17-83-C-3031

University of Maryland School of Medicine  
Baltimore, Maryland 21201

Approved for public release; distribution unlimited

The findings in this report are not to be construed as an official document of the Army position unless so designated by another authorized document.

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER IB	2. GOVT ACCESSION NO.	3. RECIPIENT'S CATALOG NUMBER
4. TITLE (and Subtitle) Development of Serologic Assays for the Diagnosis of New World Leishmaniasis		5. TYPE OF REPORT & PERIOD COVERED Annual 12-1-82 to 11-30-83
		6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s) Ronald L. Anthony		8. CONTRACT OR GRANT NUMBER(s)
9. PERFORMING ORGANIZATION NAME AND ADDRESS Department of Pathology University of Maryland School of Medicine Baltimore, Maryland 21201		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS  DAMD 17-83-C-3031
11. CONTROLLING OFFICE NAME AND ADDRESS US Army Medical Research and Development Command Fort Detrick, Frederick, Maryland 21701		12. REPORT DATE January 31, 1984
		13. NUMBER OF PAGES
14. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office)		15. SECURITY CLASS. (of this report)
		15a. DECLASSIFICATION/DOWNGRADING SCHEDULE
16. DISTRIBUTION STATEMENT (of this Report) Approved for public release: distribution unlimited		
17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)		
18. SUPPLEMENTARY NOTES		
19. KEY WORDS (Continue on reverse side if necessary and identify by block number) Leishmania Monoclonal antibodies ELISA		
20. ABSTRACT (Continue on reverse side if necessary and identify by block number) Two-hundred eighty-nine monoclonal antibodies have been generated to surface and intracellular antigens of various isolates of New World Leishmania. Concentrated efforts have been focused upon using these monoclonals to detect antigenic differences between <u>Leishmania mexicana mexicana</u> and <u>Leishmania braziliensis panamanensis</u> . Indirect immunofluorescent antibody assays have been used to demonstrate qualitative differences; enzyme linked immunosorbent assays have been used to demonstrate quantitative differences. We appear to		

have at least 6 monoclonal antibodies which can distinguish between air-dried promastigotes of the two species. Additional monoclonals react with both species but the intensity of reactivity is often significantly different. In fact, we now have some degree of confidence in our ability to differentiate L.m. mexicana from L.b. panamanensis on the basis of the quantitative differences.

REPORT NO. 1B

DEVELOPMENT OF SEROLOGIC ASSAYS FOR THE DIAGNOSIS  
OF NEW WORLD LEISHMANIASIS

ANNUAL REPORT (DECEMBER 1, 1982-NOVEMBER 30, 1983)

Ronald L. Anthony, Ph.D., Department of Pathology

January 31, 1984

US Army Medical Research and Development Command  
Fort Detrick, Frederick, Maryland 21701

Contract No. DAMD 17-83-C-3031

University of Maryland School of Medicine  
Baltimore, Maryland 21201

**Forward:**

In conducting the research described in this report, the investigator adhered to the "Guide for Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978.

Summary:

Objectives for the initial year of the contract DAMD 17-83-C-3031 included:

- (a) establishment of in-house cultures of leishmania promastigotes representative of a panel of WRAIR isolates.
- (b) preparation of monoclonal antibodies to specific antigens of those isolates.
- (c) identification and characterization of species-specific, strain-specific and stage-specific antigens.

In vitro cultures of 12 WRAIR isolates of New World Leishmania, thought to be representative of 4 species and 6 sub-species, were established in our laboratory. Adequate stocks of most isolates are now being stored in our liquid nitrogen facility.

Monoclonal hybridoma antibodies have been generated to surface and intracellular antigens of 7 isolates. Most of these monoclonals have been isotyped and they have been classified on the basis of their site of reactivity, by indirect immunofluorescence (IFA), with fixed promastigotes. One antibody, to a surface antigen of Leishmania mexicana mexicana (222B) stationary promastigotes has been used to develop an unlabeled peroxidase-anti-peroxidase technique for localization of antigen by immunoelectron microscopy.

Specificity of antibodies has been assessed by IFA and by enzyme linked immunosorbent assays (ELISA). We discovered that variations in methods of fixation of the promastigote substrate could lead to erroneous interpretations of specificity. However, many of the monoclonal antibodies do appear to have the capacity to distinguish promastigotes at the species level; e.g. Leishmania braziliensis braziliensis vs Leishmania mexicana mexicana. More extensive analyses of specificity using objective and quantitative measurements of reactivity are now in progress.

**List of Tables**

**Table 1 - Description of cell fusions**

**Page 9**

**Table 2 - Description of monoclonal antibodies**

**Page 10**

### List of Figures

- Figure 1 - Reactivity of monoclonal antibodies with 222B and 470 by enzyme linked immunosorbent assay. Page 13
- Figure 2 - Immunoelectron microscopy of monoclonal antibody 83T-9D3-B with surface antigen of 222B promastigotes. Page 15

## PROGRESS REPORT:

Statement of the problem: Development and refinement of an enzyme-linked immunosorbent assay to detect serum antibodies against species-specific, sub-species-specific and strain-specific antigens of the New World Leishmania.

Background and approach: Several significant advances have been made toward increasing the sensitivity of serologic assays for detection of the humoral antibody response in New World Leishmaniasis.<sup>1,2</sup> Nevertheless, our efforts toward increasing the specificity of these assays have been disappointing. With the exception of an indirect immunofluorescent antibody assay (IFA) which uses an amastigote as the substrate,<sup>3</sup> the IFA and the ELISA cannot distinguish between anti-leishmanial antibodies and antibodies produced in response to antigenically related organisms. Since the controlling element of specificity in the ELISA is the antigen, a prerequisite to the development of a specific serodiagnostic test for New World Leishmaniasis is development of procedures which permit the recovery of species-specific antigens on a preparative scale. We believe that these antigens can be recovered from in vitro cultures of promastigotes by using techniques of immunoadsorbent chromatography. The critical component of the immunoadsorbent will be a species-specific monoclonal antibody produced by a hybridoma. The hybridoma technique permits the generation of antibody of any desired specificity which, in turn, can be used to recover the specific antigen from a heterogeneous mixture.

1. Roffi, J., Dedet, J., Desjeux, P., and Garre, M. Detection of circulating antibodies in cutaneous leishmaniasis by enzyme-linked immunosorbent assay (ELISA). *Am. J. Trop. Med. Hyg.*, 29: 183-189, 1980.
2. Anthony, R.L., Christensen, H.A., and Johnson, C.M. Microenzyme-linked immunosorbent assay (ELISA) for the serodiagnosis of New World leishmaniasis. *Am. J. Trop. Med. Hyg.*, 29: 190-194, 1980.
3. Walton, B.C., Brooks, W.H., and Arjona. I. Serodiagnosis of American leishmaniasis by indirect fluorescent antibody test. *Am. J. Trop. Med. Hyg.*, 21: 296-299, 1972.

Results and discussion: In-house cultures of 12 WRAIR isolates were established during the first year of the contract period. These isolates included:

*Leishmania mexicana mexicana* 183B, 222B  
*Leishmania mexicana amazonensis* 303  
*Leishmania braziliensis panamanensis* 390, 424, 120, 470  
*Leishmania braziliensis braziliensis* 063, 508  
*Leishmania braziliensis peruviana* 140  
*Leishmania donovani chagasi* 111, 484

The promastigotes are maintained in 150 cm tissue culture flasks containing 100 ml Eagles MEM with 10 mM Hepes, 20% heat inactivated new born calf serum, penicillin, and streptomycin. The cultures are held in an up-right position at 26°C. Promastigotes, in the stationary phase of growth, are harvested weekly, washed in phosphate buffered saline (PBS) and stored at -70°C. Substantial stocks of most isolates have been stored in cryoprotection medium in liquid nitrogen. Isolate 063 has been discarded because of heavy yeast contamination; isolate 183B was lost prior to the generation of adequate back-up stocks.

Washed promastigotes have been used to prepare substrates for immunofluorescent microscopy. Approximately 200 slides (8 wells each) are stored at -70°C for each isolate.

Extracts of promastigotes, representative of each isolate, have been prepared by repeated freezing and thawing in PBS, followed by sonic dispersion. These extracts, standardized to contain 100 ug protein per milliliter, are used as immunogens for the BALB/c mouse and as antigens in enzyme linked immunosorbent assays (ELISA).

Fourteen successful cell fusions were performed from December 1, 1982 to November 30, 1983.

TABLE 1

Fusion number	Immunogen	Hybridomas + for antibody
83B	140	1
83H	222B	37
83I	183B	14
83L	470	26
83M	183B	1
83N	222B	7
83P	470	21
83R	470	39
83S	303	1
83T	222B	81
83U	222B	31
83V	470	1
83Y	470	10
83Z	508	19

With the exception of fusions 83T, 83U and 83V, immunogens were stationary promastigotes, frozen ( $-70^{\circ}\text{C}$ ) and thawed ( $+45^{\circ}\text{C}$ ) three times and standardized to contain 100 ug protein per ml. Promastigotes for fusions 83T and 83V were also disrupted by sonic treatment. A membrane fraction of 222B promastigotes was used as the immunogen for fusion 83U.

The fifteen monoclonal antibodies to 183B represented eight distinct specificities. An FASEB abstract of preliminary descriptions of reactivity and the use of these monoclonals to differentiate New World *Leishmania* species is appended. Since 183B is no longer available, we have discontinued our characterization of these antibodies.

The monoclonal antibody to 303 reacts with a cytoplasmic component which appears, by IFA, as sparse granules. This antibody has not been characterized.

The monoclonal antibody to 140 is an IgM which is reactive with a major constituent of the flagellum. This antibody is also strongly reactive, by ELISA, with 390 and 303. We have accumulated 5000 ml of media supporting this clone (83B7G6-A) and have recovered the immunoglobulin by precipitation in saturated ammonium sulfate. The protein concentration of our final solubilized antibody was 35 mg/ml. We intend to use this preparation to define the parameters of ELISA development.

Major efforts have been directed toward discovering monoclonal antibodies which can distinguish *L. mexicana mexicana* (222b) from *L. braziliensis panamanensis* (470). In an earlier report (1-A, August 31, 1983) we claimed that we had at least 12 monoclonal antibodies which were specific for 222B. This claim was based upon the failure of anti-222B monoclonals to react with acetone fixed promastigotes of 470 by IFA. However, we subsequently learned that the negative reactions with *L.b. panamanensis* promastigotes was a consequence of prolonged fixation. When air-dried organisms were used as the IFA substrate all 12 monoclonals were equally reactive with both isolates.



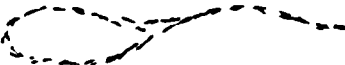
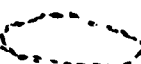

Therefore, since September, 1983, all evaluations of antibody specificity by IFA have been made on unfixed substrates. We now have 4 monoclonals which appear to satisfy our requirements of specificity.

TABLE 2

Monoclonal antibody	Immunogen	Reactivity		
		222B	470	508
83Z-9C7	508	-	+	+
83V-4G4	470	-	+	-
83P-6B4	470	-	+	-
83R-8B4	470	-	+	+

83Z-9C7 and 83R-8B4 appear to distinguish between L. braziliensis and L. mexicana, whereas 83V-4G4 and 83P-6B4 differentiate L. braziliensis panamanensis from L. braziliensis braziliensis.

The 31 monoclonal antibodies from fusion 83U, where 222B membranes were used as the immunogen, represented at least 5 different specificities (on the basis of IFA patterns).

- (a)  linear flagellum
- (b)  linear flagellum and membrane
- (c)  particulate flagellum and membrane
- (d)  particulate membrane
- (e)  linear membrane

Two monoclonal antibodies from fusion 83U failed to react with air-dried promastigotes of 470; 83U-2C6 (IFA pattern e) and 83U-6E6 (IFA pattern c). Eight monoclonal antibodies from fusion 83U reacted with 470 promastigotes but to a much lesser extent than was observed with 222B (1+ vs 4+).

The remaining 21 antibodies reacted equally with 222B and 470.

Although all monoclonals from fusions H,L,N,T, and Y reacted with both 222B and 470 promastigotes, differences in intensity of immunofluorescence were obvious. Thus, it was concluded that differentiation of species would require objective quantitative analyses such as those which can be made with an ELISA.

Medium supporting hybridomas from fusion 83L was tested for monoclonal antibodies using an ELISA. Antigens were saline sonicates of 222B and 470 promastigotes standardized to contain 30 ug protein/ml. Each well of the microtiter plate received 50 ul; 1.5 ug protein. When an optical density (OD) of greater than 0.200 was regarded as the lower limit of positivity (based upon readings of less than 0.200 with IgG from related hybridomas and unfused plasmacytoma cells) 76 hybridomas were synthesizing reactive anti-leishmanial

antibodies. Thirty-five reacted equally with 222B and 470 (difference in OD was less than 0.100); 14 reacted more strongly with 470; 26 reacted more strongly with 222B. Twenty-nine of these monoclonals (OD greater than 0.300 for either 222B or 470) have been selected for cloning and further analyses.

These results encouraged us to re-examine the reactivity of monoclonal antibodies from fusion 83T, using the ELISA instead of the IFA. Medium from unfused plasmacytomas cells (IgG-1 secretors) was used as the negative control. Results for the following three monoclonal antibodies are presented in Figure 1.

83T-2B11-C:

- anti-cytoplasmic granules of 222B promastigotes
- IgG-1,k
- negative IFA with 222B amastigotes in VERO cells
- negative IFA with acetone fixed 470 promastigotes
- positive IFA with air-dried 470 promastigotes

83T-4E3-D:

- anti-cytoplasmic granules of 222B promastigotes
- IgG-1,k
- negative IFA with 222B amastigotes in VERO cells
- negative IFA with acetone fixed 470 promastigotes
- positive IFA with air-dried 470 promastigotes

83T-9D3-B:

- anti-surface granules of 222B promastigotes
- IgG-2b,k
- positive IFA with 222B amastigotes in VERO cells
- positive IFA with acetone fixed 470 promastigotes
- positive IFA with air-dried 470 promastigotes
- positive IFA with formalin fixed 222B promastigotes
- confirmed surface reactivity by immunoelectron microscopy (Figure 2)

Figure 1

Reactivity of anti-*L. m. mexicana* monoclonal antibodies (83T9D7-B ••••; 83T4E3-D ———; and 83T2B11-C —•—•—) with antigens of 222B (●) and 470 (○) by ELISA. Wells were coated with serial dilutions of antigens ranging in concentration from 75 nanograms to 20 micrograms. Medium supporting P<sub>3</sub> cells served as the negative control (— — — —). Optical density was determined at 405 nm.

NO. 340 L410 DIETZEN GRAPH PAPER  
SEMI-LOGARITHMIC  
4 CYCLES X 10 DIVISIONS PER INCH

EUGENE DIETZEN CO.

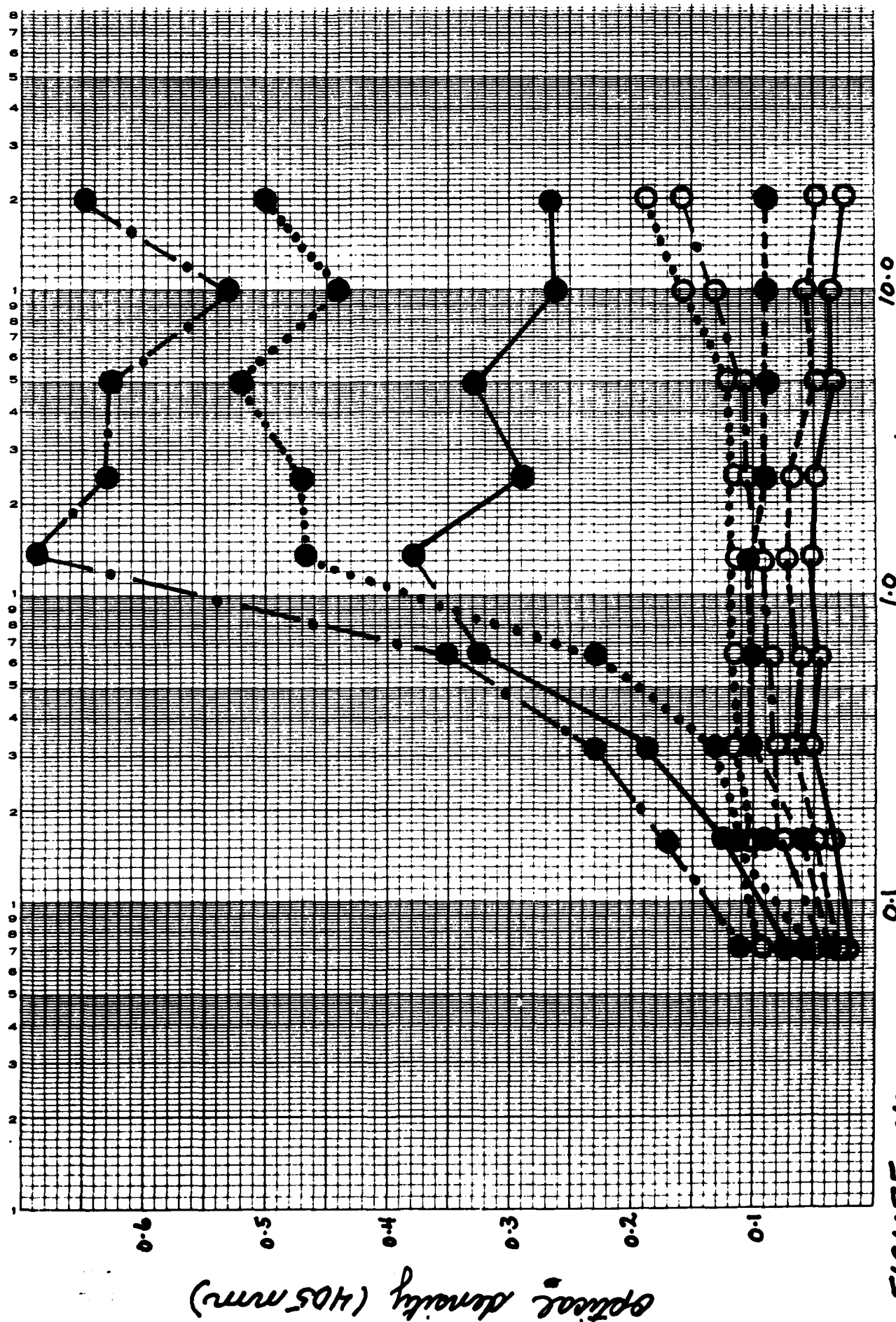


FIGURE NO. 1

Figure 2

Immunoelectron microscopy demonstrating reactivity of monoclonal antibody 83T9D7-B with surface antigens of 222B promastigotes.



**FIGURE 2**

Although similar results have been obtained with monoclonal antibodies concentrated with precipitation in saturated ammonium sulfate, additional assays utilizing several preparations of antigen are required before claims of quantitative differences can be substantiated.

Conclusions: Two-hundred eighty-nine monoclonal antibodies have been generated to surface and intracellular antigens of various isolates of New World Leishmania. Concentrated efforts have been focused upon using these monoclonals to detect antigenic differences between Leishmania mexicana mexicana and Leishmania braziliensis panamanensis. Indirect immunofluorescent antibody assays have been used to demonstrate qualitative differences; enzyme linked immunosorbent assays have been used to demonstrate quantitative differences. We appear to have at least 6 monoclonal antibodies which can distinguish between air-dried promastigotes of the two species. Additional monoclonals react with both species but the intensity of reactivity is often significantly different. In fact, we now have some degree of confidence in our ability to differentiate L.m. mexicana from L.b. panamanensis on the basis of the quantitative differences.

**-DIFFERENTIATION OF NEW WORLD LEISHMANIA SPECIES BY MEANS OF THEIR REACTIVITY WITH MONOCLONAL ANTIBODIES IN IMMUNO-FLUORESCENT ASSAYS.** Kristina M. Williams\*, Niel T. Constantine\* and Ronald L. Anthony. Department of Pathology, University of Maryland School of Medicine, Baltimore, Maryland 21201.

A panel of monoclonal antibodies has been used to differentiate seven strains of New World Leishmania: *L. braziliensis braziliensis*, *L. b. panamensis*, *L. b. peruviana*, *L. b. guyanensis*, *L. mexicana mexicana*, *L. a. amazonensis* and *L. donovani chagasi*. These monoclonal antibodies represented eight distinct specificities as evidenced by their reactivity with viable promastigotes, air-dried and acetone fixed promastigotes and intracellular amastigotes. Specific sites of reactivity included the flagellum, the flagellar pocket, intracellular granules, surface granules, surface membrane, an intracellular organelle with a size and position compatible with the kinetoplast, and an antigen with a bipolar distribution. Strain-specific antigens included both a non-granular and a granular surface component and a granular intracellular component. The kinetoplast antigen and the bipolar substance were species-specific with the exception of *L. b. peruviana*. This strain showed patterns of reactivity similar to those of the mexicana group. Antigens of the flagellum and/or flagellar pocket were common to all species and strains of Leishmania, as well as to two species of Trypanosoma. Additionally all species and strains contained at least one common surface antigen and one common intracellular antigen.

Distribution List:

Commander  
U.S. Army Medical Research and Development Command  
ATTN: SGRD-RMS  
Fort Detrick  
Frederick, Maryland 21701  
(2 copies)

Office of Research Management  
SGRD-UWZ-C  
Walter Reed Army Institute of Research  
Washington, DC 20307  
(1 copy)

Patrick B. McGreevy, CDT, MSC  
Walter Reed Army Institute of Research  
(WRAIR) Building 40  
Washington, DC 20012  
(1 copy)

